ORIGINAL ARTICLE

Clinical & Experimental
Immunology
The Journal of Translational Immunology

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immunology

Nuclear factor kappa B inhibitor suppresses experimental autoimmune neuritis in mice via declining macrophages polarization to M1 type

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Funding information
Swedish Research Council, Grant/Award
Number: 2015-03005; National Natural
Science Foundation of China, Grant/
Award Number: 81471216, 81671186 and
81671177

Abstract

Guillain–Barré syndrome (GBS) is an acute inflammatory and immune-mediated demyelinating disease of the peripheral nervous system (PNS). Macrophages play a central role in its animal model, experimental autoimmune neuritis (EAN), which has been well accepted. Additionally, nuclear factor (NF)-κB inhibitors have been used to treat cancers and have shown beneficial effects. Here, we investigated the therapeutic effect of M2 macrophage and the NF-κB pathway's correlation with macrophage activation in EAN in C57BL/6 mice. We demonstrate that M2 macrophage transfusion could alleviate the clinical symptoms of EAN by reducing the proportion of M1 macrophage in the peak period, inhibiting the phosphorylation of NF-κB p65. The NF-κB inhibitor (BAY-11-7082) could alleviate the clinical symptoms of EAN and shorten the duration of symptoms by reducing the proportion of M1 macrophages and the expression of proinflammatory cytokines. Consequently, BAY-11-7082 exhibits strong potential as a therapeutic strategy for ameliorating EAN by influencing the balance of M1/M2 macrophages and inflammatory cytokines.

KEYWORDS

BAY11-7082, EAN, GBS, macrophages, NF-κB

INTRODUCTION

Guillain–Barré syndrome (GBS) is a common autoimmune disease characterized pathologically by inflammation and demyelination in the peripheral nervous system (PNS) [1,2]. Experimental autoimmune neuritis (EAN) is a classic animal model of GBS, which has been widely used in basic research of GBS [3,4]. After activation, macrophages can be divided into proinflammatory M1 macrophages and anti-inflammatory M2 macrophages, and new research has recently found that the M3 switch phenotype also exists [5,6]. In GBS and EAN, M1 macrophages are involved in the inflammatory impairment of the myelin sheath through promoting cellular cytotoxicity and producing T helper type 1 (Th1) cytokines. M2 macrophages contribute to repairing myelin and axon by promoting the Th2 immune response and secretion of anti-inflammatory cytokines [7–9]. Several studies have demonstrated that the switch from M1 to M2 could effectively ameliorate the severity of EAN [7,10,11].

Nuclear factor kappa B (NF-κB) is an inducible transcription factor expressed in a large number of cells and is involved in immune and inflammatory responses [12]. Moreover, the

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activated form p65 of NF-κB has been observed in the sural nerve macrophages of acute and chronic inflammatory demyelinating polyneuropathy (AIDP, CIDP) and the sciatic nerves of rats with EAN, suggesting that NF-κB is attributed to inflammatory demyelination. Currently, there have been very few studies demonstrating the role of NF-κB in the course of polarized macrophages [13,14]. However, research examining the relationship between macrophages and NF-κB in this process has been controversial.

The objective of this study was to assess the effect of M2 macrophages in treating EAN and to explore the role of the NF-κB regulating macrophage subtype. Our results show that M2 macrophages were effective in treating EAN by inhibiting the activation of NF-κB p65 and the production of proinflammatory cytokines. The NF-κB inhibitor, BAY11-7082, attenuated the severity of EAN by inhibiting the NF-κB pathway and the polarization of M1 macrophages.

MATERIALS AND METHODS

Animals

Male C57BL/6J mice, 5–6 weeks old, were purchased from the Beijing Vital River Laboratory Animal Technology Co., Ltd (Beijing, China). Mice were housed in pathogen-free conditions and fed with food and water *ad libitum*. The study was approved by the local ethics committee of The First Hospital of Jilin University, Changchun, China, and all experimental procedures complied with the regulations for the management of laboratory animals in the Jilin Province (Ethical approval number: 2017-216).

Induction and clinical evaluation of EAN

EAN was induced by immunizing mice twice (days 0 and 8) by subcutaneous injection inoculum containing 150 µg P0 peptides 180–199 (GenScript, Piscataway, New Jersey, USA) and 0.5 mg *Mycobacterium tuberculosis* (strain H37 RA; Difco, Franklin Lakes, New Jersey, USA) in 25 ml saline and 25 ml Freund's incomplete adjuvant (FIA; ICN Biomedicals, Costa Mesa, California, USA) into the backs of the mice. All mice were injected with 400, 300 and 300 ng pertussis toxin (PTX; Merck, Whitehouse Station, New Jersey, USA) via tail veins on days −1, +1 and +3, respectively. Using a blinded protocol, the clinical signs of EAN were scored immediately before immunization (day 0) by two different examiners as follows until day 30: 0 = normal; 1 = less lively, reduced tonus of the tail; 2 = flaccid tail; 3 = abnormal gait; 4 = gait ataxia; 5 = mild paraparesis; 6 = moderate paraparesis; 7 = severe paraparesis; and 0.5 = intermediate clinical signs. The control group is treated in the same way as

mentioned above, but without adding P0 peptides 180–199. Experiments were replicated three times ($n = 10$).

BAY11-7082 prevented and treated EAN

BAY11-7082 (Beyotime, Nantong, China) was dissolved in 1% dimethyl sulfoxide (DMSO) in phosphate-buffered saline (PBS). EAN mice were divided into three groups (control, preventative and therapeutic). For preventative treatment, BAY11-7082 solution was administered by intraperitoneal injection (20 mg/kg) from days 2 to 8 post-immunization (p.i.) every 2 days. For therapeutic treatment, BAY11-7082 solution was administered in the same way as the preventative treatment at the same dose from the day on which the onset of symptoms (day 10) was observed to day 16 every 2 days. Control animals with EAN received the same volume of the vehicle solution (1% DMSO in PBS). Clinical signs in the mice were assessed using the same protocol described as above. Experiments were replicated three times ($n = 10$).

Flow cytometry

The spleens were removed under aseptic conditions at different phases of disease (onset, peak and recovery), and splenocytes were harvested after lysing red blood cells. Spleen mononuclear cells (MNCs) with 1×10^6 resuspended in 100 µl PBS 1% bovine serum albumin (BSA) were first stained with F4/80 (Biolegend, San Diego, California, USA), CD11b (BD Pharmingen, San Diego, California, USA), CD206 (Biolegend), Arg-1 (R&D Systems, Minneapolis, Minnesota, USA) and CD40 (BD Pharmingen), fixed and permeabilized with the fixation/permeabilization solution kit for 20 min (BD Pharmingen), and then stained with antibody for intracellular inducible nitric oxide synthase (iNOS) (eBioscience, San Diego, California, USA).

In order to observe the M2 macrophage proportion before the treated M2, the cultured macrophages were harvested and resuspended at 1×10^6 cells in PBS, then stained by F4/80-phycoerythrin-cyanin 7 (PE-Cy7) (Biolegend), CD11b-fluorescein isothiocyanate (FITC) (BD Pharmingen), CD206-BV650 (Biolegend) and arginase 1-allophycocyanin (Arg-1-APC) (R&D Systems) for 45 min at room temperature (RT). Flow cytometric data were acquired using a fluorescence activated cell sorter (FACS) Aria™ flow cytometry (BD Biosciences, San Jose, California, USA) and analyzed with FlowJo software version 7.6.1 (flowjo.com).

In FACS analysis, both F4/80 and CD11b-positive were calculated as macrophages. In macrophages, both iNOS- and CD40-positive were identified as M1 macrophages, and either CD206- or Arg-1-positive was identified as M2 macrophages.

Macrophages cultured *in vitro* and transferred to EAN mice

After lysis of the red blood cells, the collected bone marrow cells from femur and tibia were grown in complete RPMI-1640 medium (Gibco, Waltham, Massachusetts, USA) containing 10% fetal bovine serum (FBS, Sigma Aldrich, St Louis, Missouri, USA), penicillin (100 U/ml; Hyclone, Logan, Utah, USA) and streptomycin (100 U/ml; Hyclone). A total of 1×10^6 cells were seeded into 96-well plates, then stimulated with macrophage colony-stimulating factor (M-CSF) (10 ng/ml; Pepro-Tech, Rocky Hill, New Jersey, USA) for 48 h, followed by lipopolysaccharide (LPS) (10 ng/ml; Solarbio, Beijing, China) for M1 macrophage or IL-4 (10 ng/ml; PeproTech) for M2 macrophage was added and incubated for another 48 h. Thereafter, cells were washed three times by PBS to prepare for flow cytometry. The staining procedure was similar to that of the spleen cells, as mentioned above. Cultured cells (M2 macrophages) stimulated with M-CSF and IL-4, respectively, were harvested and incubated with P0 (20 µg/ml) peptide for 4 h at 37°C. After washing with PBS twice, the cells were transferred into EAN mice (0.1 ml, 1×10^6 cells for each mouse) via the caudal vein.

The other cultured cells (M1 and M2 macrophages) were added by Bay11-7082 (1 µM) and incubated for 48 h, then 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution and dimethyl sulfoxide (150 µl) were added to each hole.

Cytokine cytometric bead array (CBA)

Serum was obtained by centrifugation of blood samples at 1509*g* for 15 min at 4°C. The levels of IL-1β, IL-4, IL-6, IL-10, IL-12p70, IL-17A and TNF-α were measured using CBA kits (BD Biosciences), as per the manufacturer's instructions.

Western blot analysis

The samples of protein from sciatic nerves were loaded into 12% sodium dodecyl sulfide–polyacrylamide gel electrophoresis (SDS-PAGE) gel, then transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, Massachusetts, USA). The following antibodies were used: rabbit anti-NF-κB p65 and anti-NF-κB p-p65 monoclonal antibody (Cell Signaling Technology, Danvers, Massachusetts, USA, 1:1000); β-actin (Bioss, Beijing, China, 1:2000) and the corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies (Bioss; 1:1000). The blots were developed with the enhanced chemiluminescence kit (Amersham Imager 600; GE Healthcare, Chicago, Illinois, USA).

Densitometric analysis of Western blots were performed using ImageJ software.

Statistical analysis

All experiments were repeated three times with 10 mice in each group and data were expressed as mean ± standard error of the mean (SEM). Student's *t*-test was used for comparisons between two groups. Two-way analysis of variance (ANOVA) was used to compare the score and weight of the two groups. For all statistical analyses, the level of significance was set at $p < 0.05$.

RESULTS

Clinical course of EAN

All mice immunized with P0 peptides 180–199 in combination with complete Freund's adjuvant (FCA)-acquired EAN. In our experiment, the onset of clinical symptoms of EAN in mice occurred on day 10 p.i. After the peak phase, the severity scores of EAN decreased. On day 14 EAN mice began to recover. The other mice immunized with PBS and FCA as controls did not show symptoms of EAN (Figure 1).

M2 macrophage treatment attenuated clinical severity in EAN

On the 8th day after culture of M2 macrophages induced by M-CSF and IL-4 *in vitro*, we evaluated the proportions of macrophages and M2 macrophages by flow cytometric analysis. The proportions of macrophages and M2 macrophages
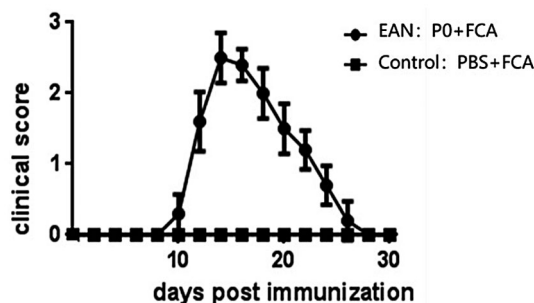
FIGURE 1 The clinical scores of experimental autoimmune neuritis (EAN) after immunization. EAN was induced in C57BL/6 mice by immunization with P0 peptides 180–199 in combination with complete Freund's adjuvant (FCA). The mice in the control group received FCA only. The statistically significant differences occurred from days 10 to 15 post-immunization (p.i.) ($p < 0.05$ at each time-point). The mean peak clinical scores were 2.6 ± 0.47 in the EAN group

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were identified as $98.48 \pm 0.189\%$ and $94.60 \pm 1.407\%$, respectively (Figure 2a).

M2 macrophages were administered via the caudal vein from days 8 to 14 p.i. (0.1 ml, 1×10^6 cells, once every 2 days) to treated EAN mice. Our results showed that that the clinical scores of EAN were significantly lighter in the M2 macrophage-treated group from days 8 to 20 p.i. compared to the control EAN group ($p < 0.05$) (Figure 2b). Additionally, the duration of symptoms was markedly shorter in the M2 macrophage-treated group than the control group receiving PBS.

## M2 macrophage treatment reduced the expression of M1 macrophages and reduced the activation of NF-κB in EAN

The flow cytometric data displayed a reduced expression of M1 macrophages (both CD40- and iNOS-positive) compared

to the control group ($p < 0.05$) and elevated expression of M2 macrophages (either CD206- or Arg-1-positive) from spleen MNCs ($p < 0.05$) (Figure 2c,d).

Western blotting results indicated that M2 macrophage treatment reduced the expression of NF-κB p-p65 in sciatic nerves of EAN mice during the peak phase compared to the control group ($p < 0.05$) (Figure 2e).

M2 macrophage treatment inhibited the levels of inflammatory cytokines in serum

When compared to the control group, a significant reduction in the expression of inflammatory cytokines, including IL-1β, IL-17A, TNF-α and IL-6, were observed in the M2 macrophage treatment group ($p < 0.05$). In contrast, the levels of the anti-inflammatory cytokines IL-4 and IL-10 in M2 macrophage treatment group tended to rise. However, there was no significant difference regarding the expression of
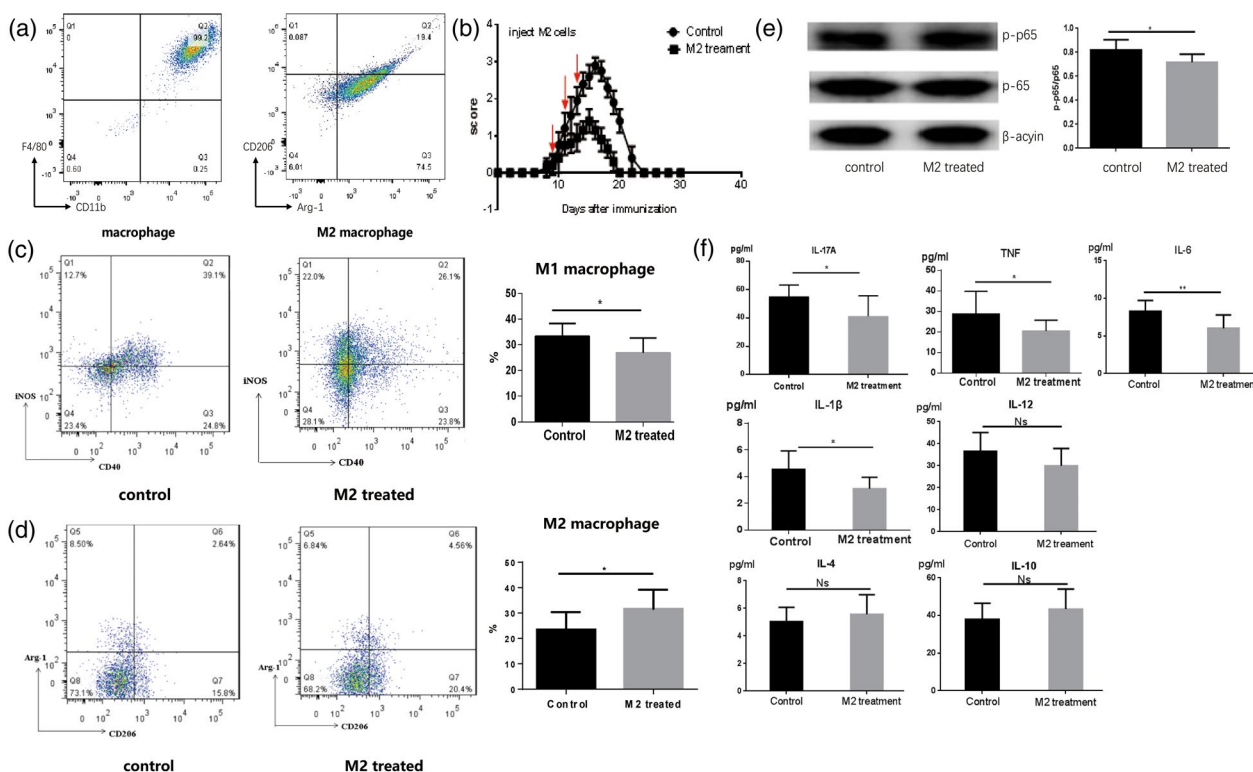
FIGURE 2 M2 macrophage-treated experimental autoimmune neuritis (EAN). (a) Macrophage colony-stimulating factor (M-CSF) and interleukin (IL-4)-induced M2 macrophages. On the 8th day after *in-vitro* culture, we evaluated the proportions of macrophages and M2 macrophages. According to flow cytometric analysis, the proportions of macrophages and M2 macrophages were $98.48 \pm 0.189\%$ and $94.60 \pm 1.407\%$, respectively. (b) M2 macrophages treatment ameliorated the severity of EAN. We administered M2 macrophages (0.1 ml, 1×10^6 cells) to EAN rats via the caudal vein from days 8 to 14. The scores for the severity of EAN decreased in the M2-treated group compared to rats in the control group ($p < 0.05$). Notably, in the M2-treated group, the duration of clinical symptoms was shorter than the control group. (c–d) The percentage of M1 macrophages was reduced and the percentage of M2 macrophages was increased in spleen mononuclear cells (MNCs) of the M2 macrophage-treated group compared to the control group. (e) M2 macrophage treatment reduced the level of p-p65 in sciatic nerves of EAN mice during the peak phase when compared to the control group. (f) The levels of interleukin (IL)-17A, IL-1β, IL-6 and tumor necrosis factor (TNF)-α decreased greatly compared to the control group. No marked differences appeared for the expression of IL-12, IL-4 and IL-10 among the two groups, but the levels of IL-4 and IL-10 increased, although there was no significance

IL-4, IL-10 and IL-12 between the control and M2 treatment groups (Figure 2f).

BAY11-7082 treatment suppressed the severity of EAN in mice

BAY11-7082 was applied using two different patterns to treat EAN mice (Figure 3a). For the preventative group, BAY11-7082 was administered from day 2 to the onset of clinical signs of EAN (day 8 p.i). For the therapeutic group, the same dose of BAY11-7082 was administered

from days 10 to 16. Our results showed that the EAN severity scores were decreased in both the preventative and treated groups with BAY11-7082 compared to control EAN groups, and the preventative group clinical score was more significant. However, the onset of EAN was delayed in the preventative group, and the duration of symptoms was shorter than both therapeutic and control groups (Figure 3a).

According to the Western blotting results, the expression of p-NF-κB p65 was inhibited in the preventative group treated by BAY11-7082 compared to the control group (Figure 3b).
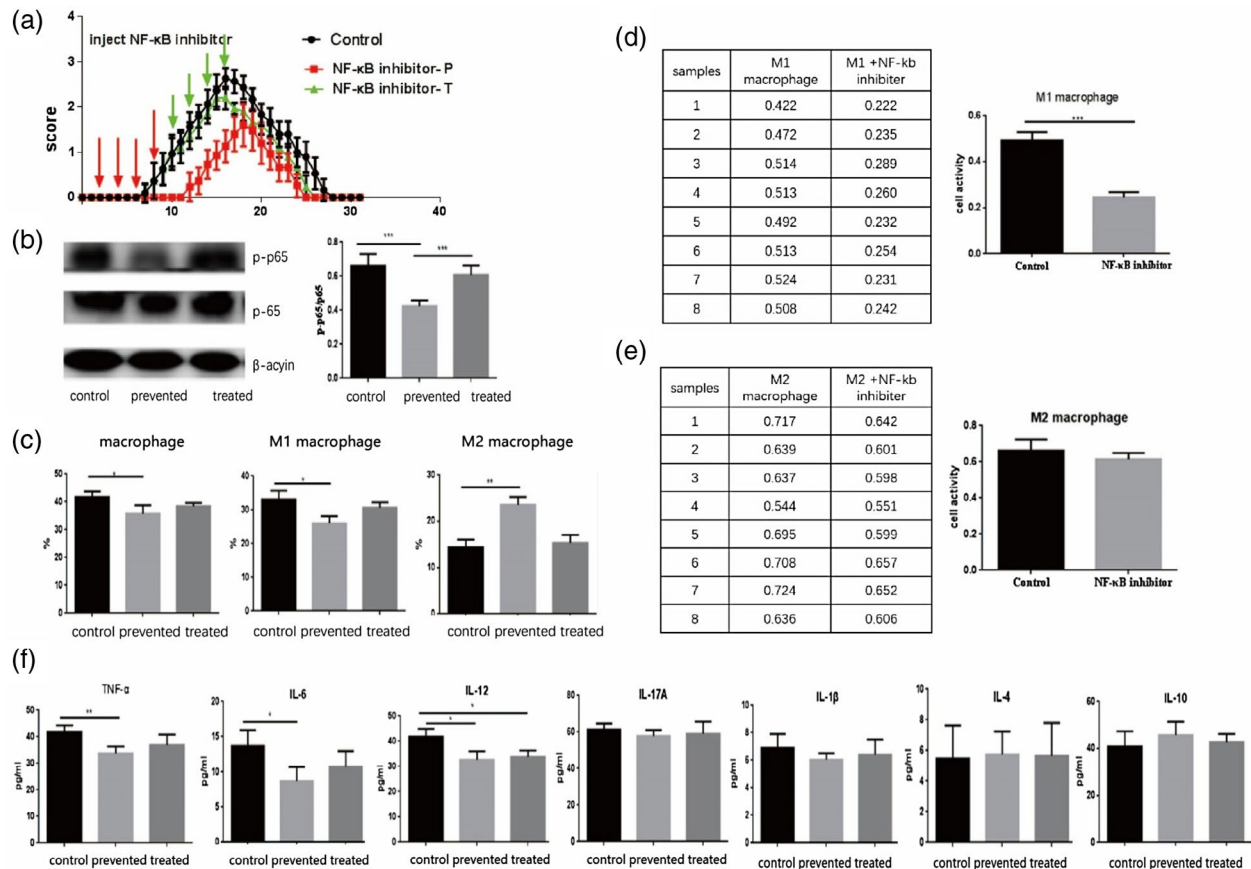
FIGURE 3 BAY11-7082 treatment ameliorated the severity of experimental autoimmune neuritis (EAN). In a preventative treatment paradigm, BAY11-7082 was administered from days 2 to 8 (20 mg/kg, once every 2 days) to EAN mice. The same dosage was applied in a therapeutic treatment paradigm from days 10 to 16. (a) EAN clinical scores were markedly better in BAY11-7082-preventative groups. The mice in the preventative group displayed notably better clinical scores from days 8 to 16 with the control group, but there no marked differences appeared for the clinical scores between the therapeutic and control groups. (b) BAY11-7082 treatment inhibited production of p-p65 in sciatic nerves of EAN mice. The BAY11-7082-preventative group had a significantly decreased expression of p-p65 compared to BAY11-7082 therapeutic and control groups. The expression of p-p65 in the BAY11-7082 therapeutic group was reduced when compared to the control group, but the differences were not significant. (c) The BAY11-7082-preventative group reduced the percentage of M1 macrophages and increased the number of M2 macrophages compared to the therapeutic and control groups. (d,e) BAY11-7082 inhibited the proliferation of M1 macrophages, whereas the proliferation of M2, despite the differences, was not significant compared to control group *in-vitro* studies. (f) The levels of tumor necrosis factor (TNF)-α, interleukin (IL)-12 and IL-6 decreased greatly in the preventative treatment group compared to the control and therapeutic treatment groups. No marked differences appeared for the expression of IL-17, IL-1β, IL-4 and IL-10 among the three groups, but the levels of IL-4 and IL-10 increased although there was no significance

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BAY11-7082 promoted M2 macrophage polarization in EAN mice

The proportion of M1 and M2 cells in spleens of EAN mice received BAY11-7082 in treatment/preventative groups was investigated via flow cytometry analysis. The results indicated that the proportion of M2 was elevated significantly in the preventative group compared to the control group ($p < 0.01$). Interestingly, the proportion of M1 was reduced significantly in the preventative group compared to the control group ($p < 0.05$), suggesting that BAY11-7082 was able to promote macrophage polarization to M2 type (Figure 3c).

To explore the mechanisms behind the effects of BAY11-7082 suppressing EAN, M1 and M2 macrophages were cultured *in vitro*, respectively, and half of them were added by BAY11-7082 as an experimental group. The activity and proliferation of M1 and M2 macrophages were evaluated by the MTT method. The results showed that BAY11-7082 inhibited the activity and proliferation of M1 macrophages significantly compared to the control cultures without BAY11-7082 ($p < 0.001$), whereas BAY11-7082 up-regulated the activity and proliferation of M2 in spite of the differences and was not significant compared to the control culture group (Figure 3d,e).

BAY11-7082 altered the expression of cytokines

The expression of inflammatory cytokines shapes the outcome of EAN [15]. We used CBA to confirm the relevant changes in cytokines of EAN after treatment with BAY11-7082; that is, the expressions of TNF-α, IL-6 and IL-12 were greatly reduced in the BAY11-7082 preventative group compared to control and therapeutic groups ($p < 0.01$) (Figure 3f). However, there were no marked differences for the expression of IL-4, IL-10, IL-1β and IL-17A among the three groups (Figure 3f). Also, differences regarding the expression of cytokines mentioned above in the control and therapeutic groups were meaningless.

DISCUSSION

In this study, we used EAN mice to explore the effects of M2 macrophages and BAY11-7082 as an NF-κB inhibitor on this disease. Our results showed that M2 macrophages ameliorated the clinical signs and reduced the duration of symptoms of EAN by inhibiting M1 macrophage and cytokine accumulation and increasing M2 macrophage polarization via inhibiting the NF-κB signaling pathway. BAY11-7082 could also diminish EAN symptoms and delay the onset of EAN by reducing M1 macrophage and proinflammatory cytokine accumulation and increasing M2 macrophage proportion in

preventive group. *In vitro*, BAY11-7082 inhibited the activity and proliferation of M1 macrophages, which was similar to other results [16]. Overall, these results demonstrated that the NF-κB signaling pathway may be involved in the pathogenesis of EAN by regulating the polarization of macrophages and inhibiting the expression of inflammatory cytokines. The results of adoptive transfer of M2 macrophages into EAN mice displayed the marked beneficial effects on EAN by inhibiting the activation of NF-κB.

Macrophages play very important roles in GBS, which are broadly divided into two phenotypes: proinflammatory macrophages (M1) and anti-inflammatory macrophages (M2) [14,17]. M1 macrophages secrete proinflammatory cytokines that cause tissue damage and disease development, whereas M2 macrophages express high levels of anti-inflammatory molecules to reduce inflammation and promote disease recovery [14,18]. Macrophages play either a pro- or anti-inflammatory role in the different stages of GBS [19]. M1 cells can promote the expression of major histocompatibility complex II (MHC-II), adhesion molecules, reactive oxygen intermediates (ROI), amplify Th1 response via exosomes and inflammatory cytokines, resulting in inflammation, broken brood–nerve barrier (BNB) and demyelination [3,20]. In contrast, M2 macrophages exert a neuroprotective role in the pathogenicity of EAN [21]. M2 macrophages may contribute to the spontaneous remyelination and regeneration of the axon [22,23] by promoting T cell apoptosis, suppressing inflammatory responses [9], clearing myelin and axonal debris [18] and inducing the secretion of anti-inflammatory cytokines such as IL-10 and transforming growth factor (TGF)-β [24].

In agreement with those findings, the present study showed that the improved outcome of EAN was associated with a higher proportion of M2 macrophages and the M2 macrophage polarization was increased in the M2 treatment group. In addition, the role of BAY11-7082 inhibiting EAN is clearly related to shifting macrophages from M1 to M2 type, as shown by the evidence, with a higher percentage of M2 macrophages and a lower percentage of M1 macrophages in EAN treated by BAY11-7082.

NF-κB is an inducible transcription factor expressed in a large number of cells and involved in immune and inflammatory responses. It plays a critical role in cell differentiation and apoptosis, as well as oncogenesis. NF-κB, as a proinflammatory signaling pathway, facilitates the inflammatory reaction by up-regulation of NF-κB target genes encoding proinflammatory cytokines, chemokines and adhesion molecules. These signals lead to the recruitment and activation of neutrophils, macrophages and leukocytes to sites of inflammation [25].

NF-κB can modulate the inflammatory response in EAN, as several studies have demonstrated that the activated p65 of NF-κB was observed in peripheral nerve macrophages in patients with AIDP or CIDP and EAN [26,27]. The activated

p65 of NF-κB in T cells and macrophages has a higher intensity at the peak of EAN than the control. The activation of NF-κB is induced by a large number of potent stimuli, such as lipopolysaccharide (LPS), TNF-α and IL-1. Activated NF-κB is responsible for the expression of many proinflammatory cytokines (TNF, IL-1, IL-6 and IL-8), chemokines [28], adhesion molecules, prostaglandins, reactive oxygen species [29] and matrix metalloproteinases [30]. P65 is required for leukocyte recruitment and macrophage activation during the onset of inflammation [31]. However, recent studies have found that NF-κB activation promoted neuronal survival by inducing the transcription of anti-apoptotic genes and a number of growth factors [32–34]. P65/RelA over-expression induces the expression of anti-apoptotic gene and protects neurons from death. NF-κB protected neurons against amyloid β-peptide toxicity, glutamate-induced toxicity and excitotoxic or oxidative stress [35].

In this work, we found that the application of BAY11-7082 could reduce the phosphorylation of NF-κB p65, and at the same time reduce M1 macrophages, increase M2 macrophages and reduce proinflammatory factors *in vivo*. *In-vitro* culture of macrophages found that BAY11-7082 could reduce the production of macrophages, especially M1 macrophages, while it had little effect on M2 macrophages. Therefore, we considered that BAY11-7082 could inhibit the phosphorylation process of NF-κB p65, reduce the production of proinflammatory cytokines and, at the same time, inhibit the formation of macrophages, especially M1 macrophages, although its effect on M2 macrophages was not clear. In addition, previous studies have reported that inhibition of NF-κB can prolong the inflammatory process and maintain leukocyte activation [36]. Therefore, the possible effects of NF-κB mediating macrophage polarization in EAN warrant further studies.

CONCLUSIONS

Elevated M2 macrophages ameliorated the clinical severity of EAN by down-regulating the activation of NF-κB p65 and the accumulation of proinflammatory M1 macrophages and cytokines. Furthermore, BAY11-7082 as an inhibitor of NF-κB attenuated EAN through mediating the phenotypical shift in macrophages from M1 to M2 cells. The anti-inflammatory effects of BAY11-7082 on EAN probably restore the balance of M1–M2 macrophages and their cytokines. The NF-κB inhibitor may be a potent candidate for the treatment of polyneurotic diseases in future.

ACKNOWLEDGEMENTS

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